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Influence of Food Restriction on Neuropeptide-Y, Proopiomelanocortin, and Luteinizing Hormone-Releasing Hormone Gene Expression in Sheep Hypothalami[1]

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ABSTRACT

Chronic food restriction decreases secretion of LH as a result of inhibitory influences on secretion of LHRH. We have previously reported that neuropeptide-Y (NPY) may directly or indirectly mediate this inhibitory effect on LHRH secretion. In the study reported here, we tested the hypothesis that long-term food restriction suppresses tonic release of LH as a result of 1) an increase in biosynthetic activity of NPY neurons in the arcuate nucleus of the hypothalamus, 2) an increase in activity of neurons that secrete β-endorphin, and 3) a decrease in biosynthesis of LHRH. To test predictions of the hypothesis, we compared levels of mRNA encoding NPY, proopiomelanocortin (POMC; the precursor peptide of β-endorphin), and LHRH, as well as tonic secretion of LH in food-restricted and well-nourished ewe lambs. Ten ewe lambs were ovariectomized at 18 wk of age and randomly assigned to receive either 100% nutritional requirements (FED; n = 5), or 30% requirements (R; n = 5) between 18 and 25 wk of age. At 25 wk of age, blood samples were taken every 10 min for 6 h and assayed for LH. The tonic release of LH in R lambs was less than that of FED lambs. Hypothalami were collected 4 days after blood sampling and sectioned at 12 μm for use in in situ hybridization. Radiolabeled molecular probes specific for mRNAs encoding NPY, POMC, or LHRH were hybridized to hypothalamic tissue sections. Levels of NPY mRNA were 88% greater in R vs. FED lambs ($p < 0.01$), whereas levels of POMC mRNA were 52% lower in R vs. FED lambs ($p < 0.01$). Mean cellular levels of mRNA encoding LHRH did not differ between R and FED lambs. Although the inhibitory effects of food restriction on secretion of LH may be mediated by NPY, it is unlikely that β-endorphin is a major inhibitory influence on LHRH neurons during undernutrition. Our results also provide further evidence that inhibition of LH secretion in food-restricted lambs does not involve decreased biosynthesis of LHRH.

INTRODUCTION

Chronic food restriction delays the onset of puberty and causes cessation of reproductive cycles in females of several mammalian species (human [1, 2], sheep [3, 4], cattle [5, 6], pig, [7–9], rat [10, 11]). The primary reason for impaired reproduction in food-restricted females is a decrease in pituitary secretion of LH as a result of central inhibitory influences on secretion of LHRH [12–17]. Several neuronal systems have been shown to have inhibitory effects on secretion of LHRH [18]; however, it is not clear which system(s) mediates the inhibitory effects of food restriction.

This study focuses on two possible systems previously implicated in the control of LH release. First, we have previously observed central inhibitory effects of neuropeptide-Y (NPY) on secretion of LH in ewes, and elevated concentrations of NPY in the central nervous system of food-restricted ewe lambs [19]. Morphological studies have reported that NPY immunoreactive neurons synapse on LHRH neurons in the preoptic area [20, 21]. Together, these data suggest that NPY may be an inhibitory link between food restriction and LHRH secretion. Second, opioid peptides have also been shown to inhibit pulsatile secretion of LH in sheep [22–25], and morphological evidence suggests that these effects are direct [26, 27]. The possible involvement of opioid inhibition of LH during food restriction has been studied in several species through use of the opioid receptor antagonist naloxone [28–31]. In each of those studies, treatments with naloxone did not result in an increased release of LH during food restriction, suggesting that an inhibitory opioid tone was not a cause of suppressed LH release during food restriction. However, since a direct measure of opioid neuronal activity was not examined in those studies, the possibility existed that the biosynthetic activity of opioid neurons and additional inhibitory inputs (e.g., NPY) both increased during chronic food restriction. Therefore, the consequence of blocking the action of just one input (e.g., via naloxone) would not necessarily result in restoration of LH release. Alternatively, it is possible that opioids regulate NPY neuronal activity [32], and a suppression of opioid inhibition may contribute to elevated NPY biosynthesis observed during food restriction.

In this study we used in situ hybridization histochemistry to test the hypothesis that long-term food restriction suppresses tonic release of LH as a result of 1) an increase in biosynthetic activity of NPY neurons in the arcuate nucleus (ARC) and median eminence (ME), 2) an increase in activity of neurons in the ARC that secrete β-endorphin, and 3) a decrease in biosynthesis of LHRH. Based on the premise that neuropeptide biosynthesis is coupled with release

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[33, 34], this hypothesis predicts that hypothalamic levels of mRNA for NPY and proopiomelanocortin (POMC) will be elevated in long-term food-restricted ewe lambs, coincident with decreased levels of LHRH mRNA and decreased tonic secretion of LH. To test these predictions, we compared tonic secretion of LH as an accurate indicator of tonic LHRH release [35, 36], and cellular levels of mRNA for LHRH, NPY, and POMC in hypothalami of food-restricted vs. well-fed female lambs.

TABLE 1. Experimental diets (100% dry matter), daily nutrient intake required for 30 kg replacement ewe lambs (Nutritional Research Council, 1985), and nutrients provided to well-nourished (FED) and food-restricted (R) treatment groups.

Ingredient	Composition (%)
Corn	49.25
Cottonseed hulls	40.60
Soybean meal	8.19
Dicalcium phosphate	0.36
Limestone	0.83
Trace mineral salt	0.47
Urea	0.30
Metabolizable Energy (Mcal/kg)	2.7
Crude Protein (%)	11.0
Calcium (%)	0.48
Phosphorus (%)	0.29

Group	Dry matter intake (kg)	Metabolizable energy (Mcal)	Crude protein (g)
Daily requirement	—	2.80	185
FED	1.7	4.59	187
R	0.3	0.81	33

MATERIALS AND METHODS

Animals and Tissue Collection

Ten spring-born ewe lambs were housed outdoors in Missouri under naturally occurring photoperiod from April to November. Lambs were ovariectomized at 18 wk of age and had an average body weight of 23.3 ± 0.45 kg. Lambs were randomly assigned to groups receiving one of two diets (Table 1): > 100% Nutritional Research Council (1985) requirements for 30-kg growing replacement ewe lambs (FED; $n = 5$), or < 30% NRC requirements (R; $n = 5$). Lambs were group-fed between 18 and 25 wk of age. At 25 wk of age, blood samples were taken every 10 min for 6 h, and serum was assayed for LH. Four days after blood collection, brains were collected after rapid decapitation, and hypothalami were dissected and frozen in crushed dry ice. Tissue was stored at −80°C and allowed to warm to −20°C before sectioning at 12-μm intervals by means of a cryostat. Every fifth coronal hypothalamic section from the optic chiasm to the caudal end of the ME was collected onto positively charged glass slides, then stored at −80°C until hybridized for mRNAs encoding NPY, POMC, or LHRH.

RIA of LH

Blood samples were allowed to clot at room temperature for 1 h and stored at 4°C for 2–8 h before centrifugation. Serum was decanted and stored at −20°C until assayed. Serum concentrations of LH were assayed in duplicate 100-μl samples by the methods of Fogwell and coworkers [37], with use of RAoLH TEA #35 ovine LH antiserum [38]. The intraassay coefficient of variation was 6.3% ($n = 4$), with a minimum detectable concentration of 60 pg/tube (0.6 ng/ml).

In Situ Hybridization Procedures

A pSP62-PL vector containing a 591-base fragment of human NPY cDNA (provided by Dr. Carolyn Minth, Wright State University, Dayton, OH [39]) was linearized with *Bam*HI (Promega Biotec, Madison, WI) and used as a transcription template to synthesize a cRNA probe (NPY-cRNA). The NPY-cRNA probe was synthesized by incubating 1 μg linearized DNA template with the following at 37°C: 250 μCi ^{35}S-cytidine triphosphate (Amersham Corp., Arlington Heights, IL; dried down in bottom of sterile microfuge tube); 2 μl 5-strength transcription buffer (Promega); 1 μl 100 mM dithiothreitol (DTT; Fisher Scientific Co., Pittsburgh, PA); 0.5 μl RNasin (Promega); 2.5 μl each of 10 mM ATP, uridine triphosphate, and guanidine triphosphate (Promega); and 0.5 μl of SP6 polymerase (Promega). After a 30-min incubation, 5 μl 1 M Tris HCl (pH 8.0), 1 μl 1 M MgCl$_2$, 0.5 μl yeast tRNA (25 μg/μl; Boehringer-Mannheim, Indianapolis, IN), 0.5 μl RNasin (40 U/μl), and 2 μl DNase-I (1 U/μl; Promega) were added and incubated at 37°C for 30 min to remove the DNA template. The labeled probe was purified in a 1-ml Sephadex G-50 column (made from a 1-cc syringe) by centrifugation.

Before hybridization, every third tissue section through the ARC/ME (corresponding to every 180 μm) of each hypothalamus was prepared as described previously [40]. Briefly, tissue sections were thawed at room temperature for 10 min before fixation, which consisted of immersion into 4% formaldehyde in 0.12 M PBS (pH 7.4) for 5 min followed by quick rinses in double-strength NaCl/sodium citrate (double-strength SSC; single-strength SSC = 0.15 M NaCl/0.015 M sodium citrate; pH 7.2). Sections were then acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine-HCl/0.9% NaCl (pH 8.0) for 10 min at room temperature. After acetylation, sections were dehydrated and delipidated in a series of ethanol and chloroform baths, then dried prior to hybridization. Hybridization was performed overnight at 50°C under parafilm coverslips. Each section was covered with 50 μl buffer composed of 50% formamide, 5 M NaCl, 1 M Tris-HCl (pH 8.0), 0.5 M EDTA, 100-strength Denhardt's solution (2% Ficoll, 2% polyvinylpyrrolidone, and 2% BSA), 500 μg/ml yeast tRNA, 10 mM DTT, and 10% dextran sulfate (M_r 500 000). The buffer also contained approximately 10^6 cpm of ^{35}S-labeled probe per 50 μl. After the overnight hybridization, sections were washed

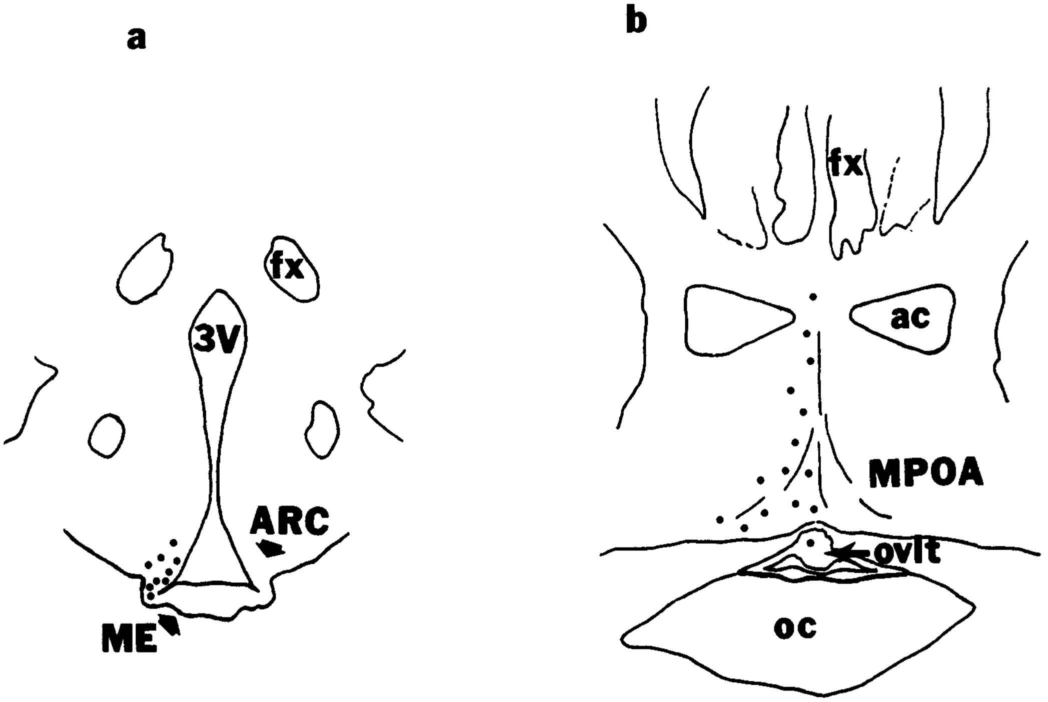

FIG. 1. Illustration of coronal hypothalamic sections through the a) ARC and ME, and b) MPOA in sheep. Dots represent the distribution of neurons labeled for a) NPY mRNA and POMC mRNA, and b) LHRH mRNA. fx, fornix; 3V, third ventricle; oc, optic chiasm; ac, anterior commissure; ovlt, organum vasculosum of the lamina terminalis.

in four 15-min changes of 4-strength SSC followed by an RNAse treatment (0.5 M NaCl, 10 mM Tris [pH 8.0], 1 mM EDTA [pH 8.0], 20 μg/ml RNAse A [Sigma Chemical Co., St. Louis, MO]) and incubated for 30 min at 37°C. The final washes included 15 min in each of the following: double-strength, single-strength, and 0.5-strength SSC, each containing 10 mM DTT, followed by 30 min in 0.1-strength SSC with 10 mM DTT at 55°C. After a series of dehydration steps in absolute ethanol, a brief water rinse, and a 3-min rinse in 70% ethanol, slides were dried. To obtain film autoradiographs, the tissue sections were exposed to Fuji x-ray film for 2 wk.

The 48-base oligonucleotide DNA probe used to detect mRNA encoding POMC was complementary to a portion of the POMC coding region of rat POMC cDNA (amino acids 96–111 [41]). The DNA probe used to detect LHRH mRNA [42] was complementary to the LHRH coding region of mouse LHRH cDNA (bases 1677–1724). Both oligoprobes were synthesized by means of an Applied Biosystem 380A DNA synthesizer (Applied Biosystems, Foster City, CA). The oligoprobes were radiolabeled at the 3′ end by incubation (37°C; 30 min) with [^{35}S]-dATP (1 μM; New England Nuclear Corp., Boston, MA) and terminal deoxynucleotidyl transferase (100 U; New England Nuclear) as previously described by Petersen and coworkers [40].

Before hybridization, every third section through the ARC/ME and the medial preoptic area (MPOA) was fixed, dehydrated, and delipidated as described above. Each section was covered with 50 μl buffer consisting of 50% formamide, 4-strength SSC, 10% dextran sulfate (M_r 500 000), 250 μg/ml yeast tRNA, 500 μg/ml sheared single-stranded salmon sperm DNA (Boehringer-Mannheim), and single-strength Denhardt's solution. The buffer also contained approximately 1×10^6 cpm of ^{35}S-labeled probe per 50 μl. After the overnight hybridization at 37°C, sections were washed in four 15-min changes of 50% formamide/double-strength SSC at 40°C followed by two changes of single-strength SSC for 1 h each at room temperature. After a brief water rinse followed by a 5-min rinse in 70% ethanol, sections were dried. The sections hybridized with probe for POMC mRNA were placed against film and exposed for 2 wk before developing. Slides hybridized with the LHRH mRNA probe were dipped in Kodak NTB3 photographic emulsion and stored at 4°C for 2 wk. The emulsion-coated slides were developed, then counterstained with toluidine blue dye and coverslipped for microscopic visualization of individual neurons.

Image Analysis

Resulting autoradiographs were analyzed by means of a Bioquant MEG IV system coupled to a Dage 70 camera and a Leitz Laborlux microscope as previously described [40]. Briefly, the system was calibrated to measure film density by setting of appropriate thresholds for highlighting signal. For analysis of film autoradiographs, the ARC/ME region (Fig. 1) was highlighted, and the average gray level of the highlighted pixels was determined and then corrected for nonspecific signal, resulting in a specific density. The specific densities were used to determine relative levels of hy-

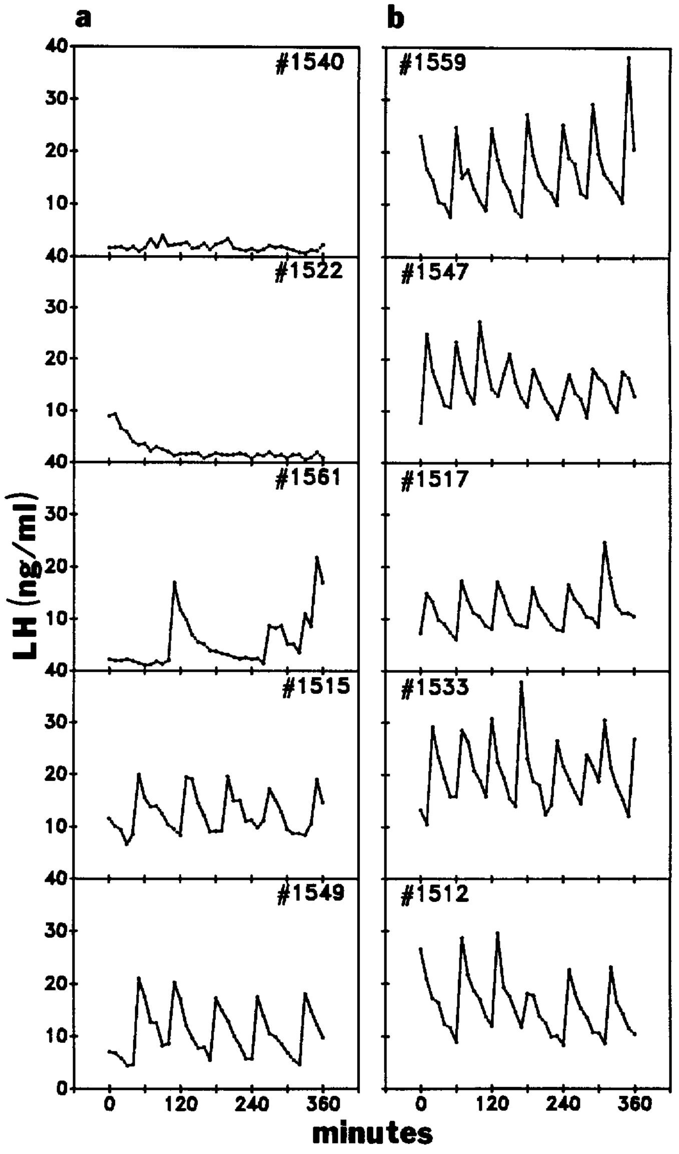

FIG. 2. Individual profiles of concentrations of LH in samples taken every 10 min for 6 h for (a) R and (b) FED lambs.

bridization to endogenous mRNAs. An average of 21 ± 2.4 autoradiograph sections for NPY mRNA and 21.1 ± 0.9 autoradiograph sections for POMC mRNA were analyzed for each animal. Single-cell analysis of LHRH neurons was performed by use of a 40× objective with a 2.5× magnification changer on the Leitz microscope for maximal resolution. A threshold was set so that silver grains were highlighted on a digitized image; then the area occupied by grains was determined. An average of 104.0 ± 11.4 LHRH cells in sections through the MPOA containing the region of the organum vasculosum of the lamina terminalis (ovlt; Fig. 1) were analyzed for each animal.

Statistical Analyses

Differences in average regional levels (expressed as specific film density of film autoradiographs) of mRNAs encoding NPY or POMC in the ARC and ME were compared in FED and R ewes by analysis of variance [43]. Variations due to effects of diet (FED or R), animal, autoradiograph within animal, and side of the hypothalamic section (left or right) were included in the model. Differences in number of pixels per neuron probed for mRNA encoding LHRH were compared in FED and R ewes using one-way analysis of variance. Variations due to effects of diet, animal within diet, and individual neurons were included in the model. Mean concentrations of LH were determined for each animal and correlated with measurements from NPY, POMC, and LHRH mRNA hybridizations. All means reported are least squares means with corresponding standard error (SEM).

RESULTS

As a result of food restriction, growth of R lambs was slowed, and their body condition was less than that of FED lambs at the time of blood sampling and decapitation. Mean body weights were lower in R than in FED lambs (19.7 vs. 31.2, SEM = 1.4 kg, respectively; $p < 0.01$). Pulsatile release of LH was suppressed (< 3 pulses observed in 6 h) in 3 of 5 R lambs compared to FED lambs (Fig. 2), and mean concentrations of LH were lower in R than in FED lambs (Table 2). Two of the 5 R lambs (1515 and 1549) did not appear to respond to food restriction with a dramatic suppression in tonic release of LH, although their LH pulse patterns did appear intermediate between the 3 of 5 R lambs with very few pulses of LH, and the FED lambs with high LH pulse frequency and amplitude patterns (Fig. 2). Interestingly, in addition to the intermediate LH patterns, the 2 of 5 R lambs (1515 and 1549) also displayed intermediate levels of NPY and POMC mRNAs (Fig. 3).

Cell-specific hybridization was detected for NPY and POMC mRNA in the ARC/ME (Fig. 1a) and in no other hypothalamic regions, corresponding to localization of cell bodies immunoreactive to anti-NPY in sheep [44] and POMC in rats [45] and cows [27]. Representative autoradiographs from coronal hypothalamic sections in the ARC and ME hybridized to mRNA encoding NPY or POMC are shown in Figures 4 and 5, respectively. Average specific film density of signal for NPY mRNA in the ARC/ME region in R lambs was 88% greater than that in FED lambs ($p < 0.01$; Table 2), while average film density for POMC mRNA in R lambs was 52% less than that in FED lambs ($p < 0.01$).

Cell-specific hybridization for LHRH mRNA was detected throughout the MPOA in a distribution (Fig. 1b) similar to the distribution of LHRH immunoreactive cells reported by Lehman and coworkers [46]. Figure 6 shows cell-specific hybridization of the cDNA probe for LHRH mRNA. The average number of neuron cell bodies detected per animal did not differ between FED and R lambs (118.2 vs. 89.8, SEM = 15.51). Average cellular levels of mRNA for LHRH in the MPOA did not differ between FED and R lambs (Table 2).

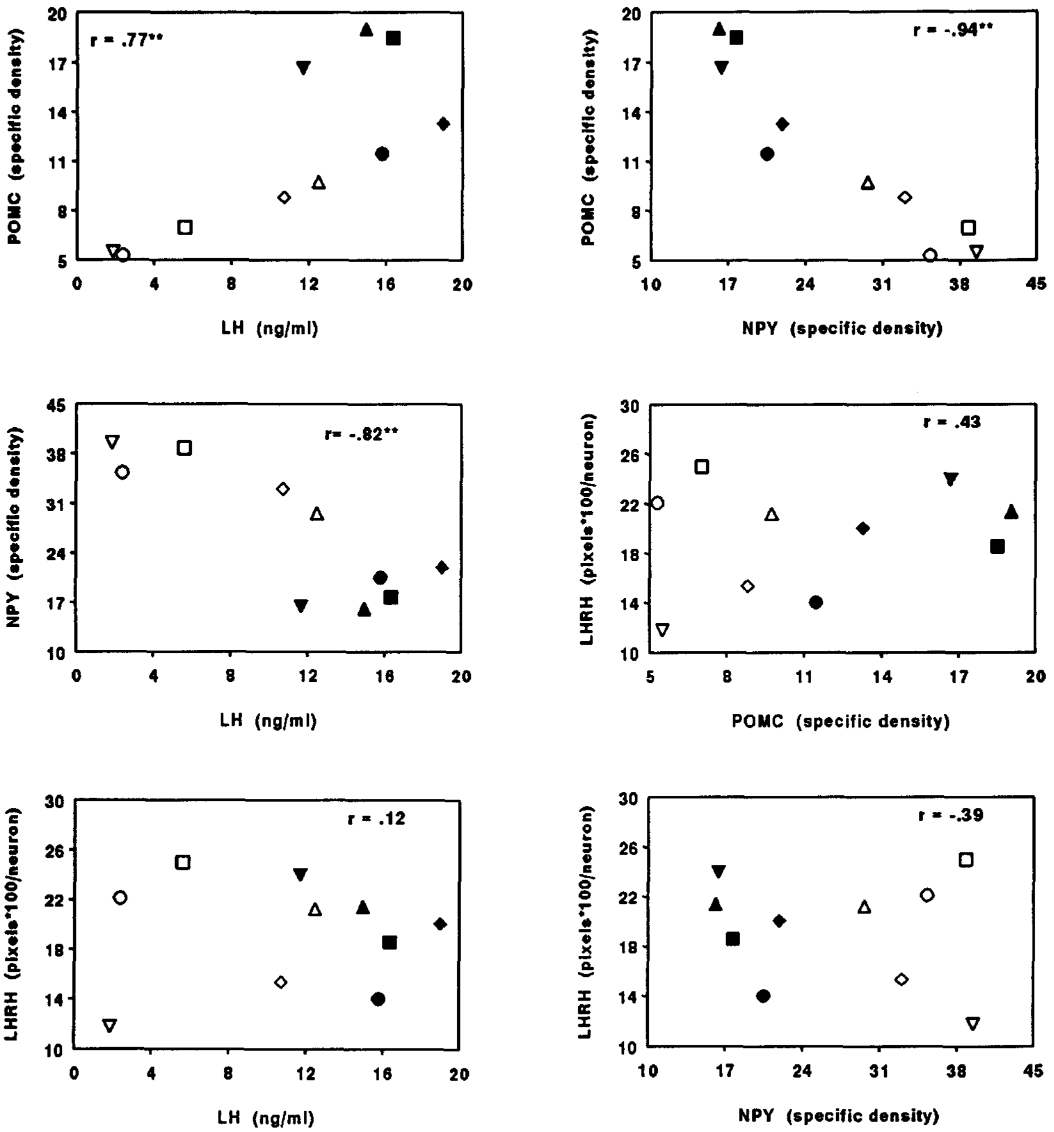

FIG. 3. Relationships among individual (n = 10) mean concentrations of LH (ng/ml), individual mean levels of NPY mRNA (specific density) and POMC mRNA (specific density), and individual mean levels of LHRH mRNA (pixels*100/neuron) for each of the lambs studied. Solid symbols represent FED lambs (▲-1547, ●-1512, ▼-1517, ◆-1533, and ■-1559); open symbols represent R lambs (△-1515, ○-1522, ▽-1540, ◇-1549, and □-1561). $^{**}p < 0.01$ denotes a significant Pearson correlation coefficient.

Correlations between individual mean concentrations of LH and individual mean levels of mRNAs encoding NPY, POMC, or LHRH are depicted in Figure 3. There was a significant negative correlation between mean concentration of LH and level of NPY mRNA and also between level of NPY mRNA and POMC mRNA. In contrast, there was a significant positive correlation between LH and POMC mRNA. Level of LHRH mRNA did not correlate to any of the other measurements.

DISCUSSION

The present study demonstrates that increased levels of NPY mRNA and decreased levels of POMC mRNA in the ARC and ME of R lambs are coincident with suppressed tonic secretion of LH, but no change was observed in LHRH mRNA. The significant correlations between NPY and POMC biosynthetic activity and secretion of LH suggest that these systems may be functionally linked. Furthermore, we argue that the "link" functions in a continuum, as inferred from the two restricted lambs (1515 and 1549) that had intermediate levels of LH, given their consistent intermediate expression of mRNAs encoding NPY and POMC. These findings are consistent with our hypothesis that chronic undernutrition increases the activity of hypothalamic NPY neurons coincident with decreased secretion of LH in food-restricted lambs. We reject the parts of our hypothesis, however, predicting that food restriction would result in

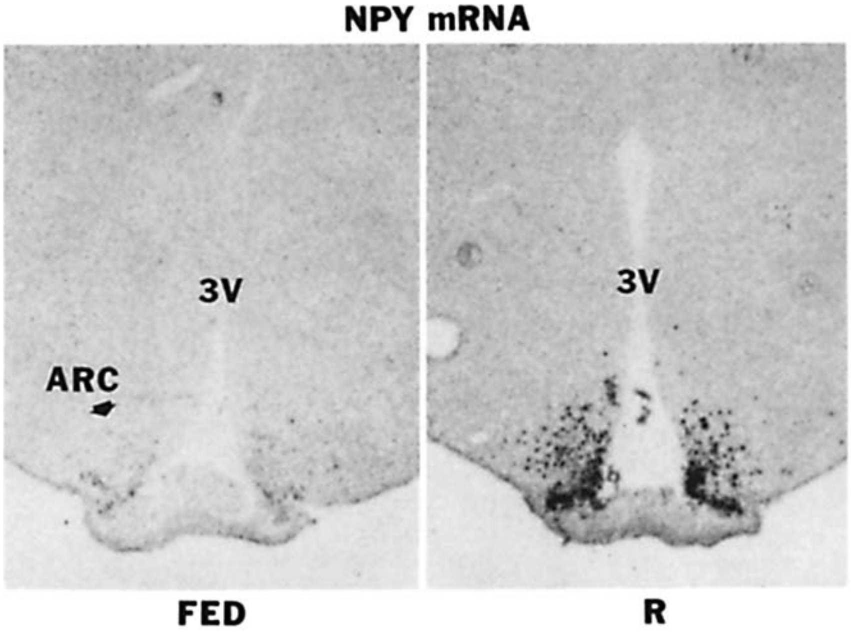

FIG. 4. Representative autoradiographs of coronal hypothalamic sections through the ARC and ME at the base of the third ventricle (3V) from R and FED lambs. Sections were hybridized to probe specific for mRNA encoding NPY.

increased activity of β-endorphinergic neurons and decreased biosynthetic activity of LHRH neurons.

Our finding that NPY mRNA was elevated and POMC mRNA was suppressed in food-restricted lambs, however, is consistent with several other studies that report elevated NPY mRNA or peptide content [19, 47–51], and decreased POMC mRNA [47] in the central nervous system during food restriction. Increased NPY immunoreactivity in response to food restriction has been previously reported in sheep [19, 50] and coincides with decreased tonic release of LH [50]. These studies may be relevant to human pathophysiology because concentrations of NPY in the cerebrospinal

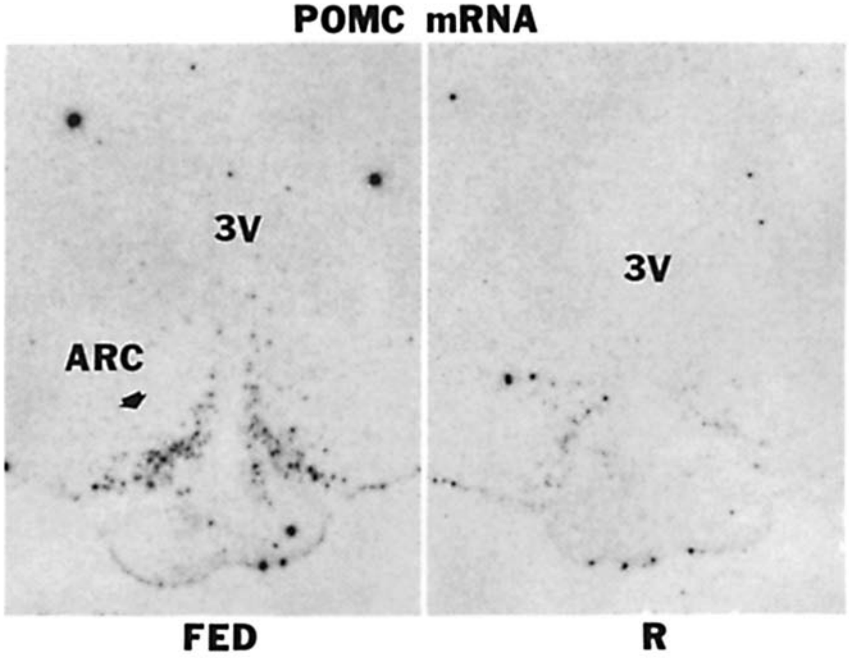

FIG. 5. Representative autoradiographs of coronal hypothalamic sections through the ARC and ME the base of the third ventricle (3V) from R and FED lambs. Sections were hybridized to probe specific for mRNA encoding POMC.

TABLE 2. Mean concentrations of LH, mean regional levels of mRNA for NPY and POMC, and mean cellular levels of LHRH mRNA in R (n = 5) and FED (n = 5) ewe lambs.

	FED	R	S.E.M.
LH (ng/ml)	15.82	6.61**	4.19
NPY mRNA (corrected density)	20.52	36.02**	6.48
POMC mRNA (corrected density)	16.40	6.07**	5.87
LHRH mRNA (pixels)	1997	1922	1460

**Mean differ ($p < 0.01$).

fluid of anorexic women, who are often amenorrheic, were shown to be elevated [49], while concentrations of β-endorphin were low [52] when compared to measurements taken after recovery from this form of undernutrition.

The effects of central injection of NPY on tonic secretion of LH varies with the presence or absence of the ovary/estradiol in some species (rat [53, 54]; rabbit [55]; macaques [56]). In sheep, however, central injection of NPY in ovariectomized ewes decreased tonic release of LH [57], and this occurred regardless of estradiol treatment [19]. Because morphological studies have shown that NPY neurons synapse on LHRH neurons in the MPOA [20, 21], and the biosynthetic activity of NPY neurons is elevated during food restriction, it is likely that NPY neurons are an important neuromodulatory link between nutritional status and activity of LHRH neurons.

It is unlikely that POMC-synthesizing neurons directly suppress LHRH secretion during food-restriction because mRNA encoding POMC in the ARC and ME was lower in R lambs than in FED controls. In fasting female rats treated with naloxone, an increase in tonic release of LH was realized [58], suggesting that increased opioid tone may contribute to nutritionally induced hypogonadotropism. However, similar studies in undernourished sheep [28, 29], gilts [30], and women [31] report no effects of naloxone on tonic secretion of LH, suggesting that opioid pathways do not contribute a major inhibitory influence on LHRH release during long-term undernutrition. We suggest that the decreased biosynthetic activity of POMC neurons we observed in food-restricted lambs provides further evidence that inhibitory opioid input probably does not play a major role in suppression of LH during chronic undernutrition.

Our finding of a very strong ($p < 0.01$) negative correlation (-0.94) between measurements of NPY mRNA and POMC mRNA may indicate a possible opioid modulation of NPY neuronal activity, or vice versa. This was proposed previously by Sahu and coworkers [32], who reported that treatment with an opiate receptor antagonist (naloxone) increased NPY concentrations in the ARC, ME, and MPOA in rats. Thus, the possibility exists that undernutrition suppresses opiate tone, thereby increasing the activity of NPY neurons.

Levels of mRNA encoding LHRH were not influenced by food restriction and did not correlate with mean concentration of LH; therefore changes in biosynthesis of LHRH are not likely to be the reason for suppressed LH release. This explanation is consistent with a report by Ebling and coworkers [28], who measured LHRH content in extracts of the medial basal hypothalamus and found no difference between food-restricted and well-fed ewe lambs. Tatman and coworkers [59] reported that LHRH content in the stalk ME was higher in nutrient-restricted ovariectomized ewes char-

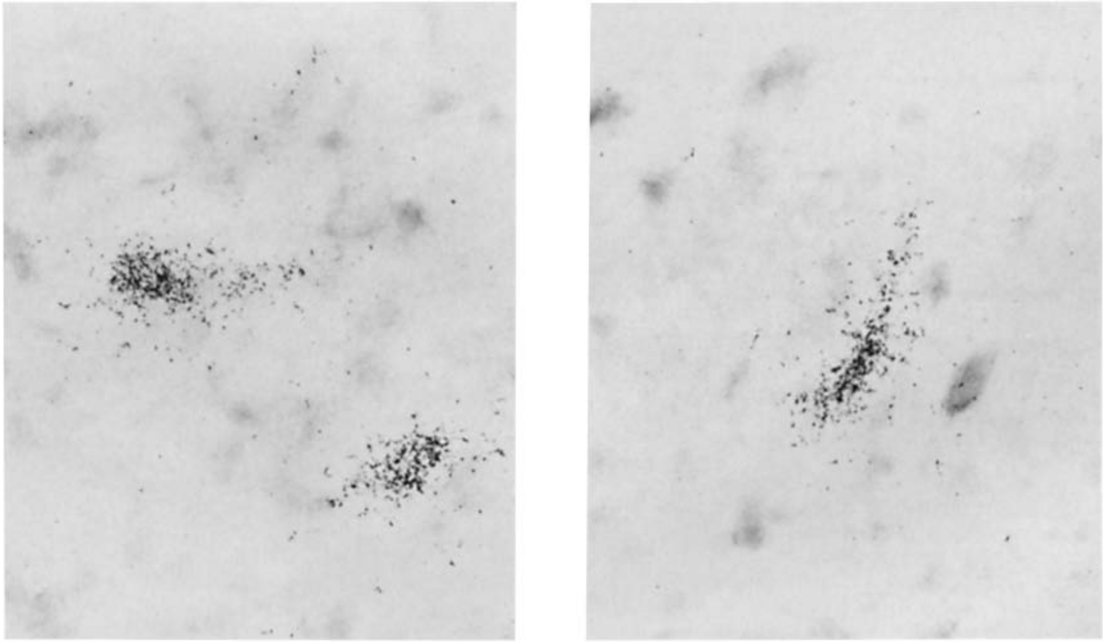

FIG. 6. Bright-field photomicrographs of probe hybridized to LHRH mRNA in individual neurons within the MPOA region of sheep hypothalamus.

acterized as very thin compared to adequately fed ewes. Direct measurement of LHRH in portal blood has shown that individual pulses of LH are almost always preceded by a pulse of LHRH [35, 36], and it has recently been demonstrated that this close association between LHRH pulses and LH pulses is maintained in food-restricted ewe lambs [60]. The inability to detect changes in LHRH content [28] and LHRH mRNA levels in food-restricted lambs could have several explanations: post-transcriptional processes are affected differentially during food restriction, rate of LHRH or LHRH mRNA degradation is altered during food restriction, or the methods used to measure LHRH content and LHRH mRNA were not sensitive enough to detect what could be very small changes associated with tonic LHRH secretion. It is likely that central mechanisms controlling release of LHRH from nerve terminals, independent of biosynthesis of LHRH, limit LHRH [60] and LH secretion in food-restricted lambs.

In summary, these results provide further evidence that the effects of food restriction on secretion of LHRH are mediated by NPY, and that β-endorphinergic neurons do not provide a significant inhibitory input to LHRH neurons during undernutrition. Alternatively, POMC-synthesizing neurons may inhibit NPY neurons, and during food restriction, a decrease in this inhibitory input may allow for increased levels of NPY biosynthesis, which either directly or indirectly influences LHRH release, but not biosynthesis. Our results also provide further evidence that inhibition of LH secretion in food-restricted lambs does not involve decreased biosynthesis of LHRH.

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